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Laboratory and Clinical Experience with a Monoclonal Antibody-Based Radioimmunoassay for Serum Total Thyroxine

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Summary: The performance characteristics and diagnostic value of a monoclonal antibody-based radioimmunoassay for serum total thyroxine (Mallinckrodt) are described. Between-batch precision (coefficient of variation) was 10.4% at 87 nmol/l and 3.3% at 185 nmol/l. *Scatchard* analysis revealed a linear plot with a K_a of 5.4×10^8 l/mol.

Sensitivity was 4.5 nmol/l of thyroxine. An association study showed that the assay reached equilibrium well within the specified incubation time. Cross-reaction of triiodothyronine and reverse triiodothyronine in the assay was 0.6% and 25.0% respectively. Analytical recovery was 91–110%. Linearity was well demonstrated but dilutions of a high concentration of thyroxine in serum did not parallel the standard curve. The correlation coefficient for comparison with a polyclonal antibody assay was 0.95 for 83 patients. The diagnostic accuracy of the monoclonal antibody assay was adequate for most patients with thyroid disease, pregnant women, oral contraceptive users and subjects on thyroxine-replacement therapy. Measurement of total thyroxine by a monoclonal antibody-based method shows no definite advantage over the conventional polyclonal antibody assay.

Erfahrungen in Laboratorium und Klinik mit einem Radioimmunassay für das gesamte Thyroxin im Serum auf der Grundlage eines monoklonalen Antikörpers

Zusammenfassung: Die Charakteristika der Durchführung und der diagnostische Wert eines Radioimmunassay mit monoklonalem Antikörper für das gesamte Thyroxin im Serum (Mallinckrodt) werden beschrieben. Die Impräzision von Serie zu Serie beträgt als Variationskoeffizient 10,4% bei 87 nmol/l und 3,3% bei 185 nmol/l. Die *Scatchard*-Analyse ergab einen linearen Verlauf mit einem Wert für K_a von $5{,}4 \times 10^8$ l/mol.

Die Empfindlichkeit betrug 4,5 nmol/l Thyroxin. Eine Bindungsstudie ergab, daß das Gleichgewicht innerhalb der angegebenen Inkubationszeit völlig erreicht wird. Die Kreuzreaktionen von Triiodthyronin und reverse Triiodthyronin betrugen 0,6 bzw. 25,0%. Die analytische Wiederfindung betrug 91–110%. Es konnte völlige Linearität nachgewiesen werden, jedoch ergaben Verdünnungen einer hohen Konzentration von Thyroxin im Serum keinen parallelen Verlauf mit der Standardkurve. Der Korrelationskoeffizient für ein Vergleichsverfahren mit polyklonalem Antikörper betrug $r = 0{,}95$ ($n = 83$ Patienten). Die diagnostische Richtigkeit des Radioimmunassay mit monoklonalem Antikörper war für die meisten Patienten mit Schilddrüsenerkrankungen, für Frauen, die orale Kontraceptiva einnahmen oder schwanger waren, sowie Personen unter Thyroxin-Substitution genügend. Die Bestimmung des Gesamtthyroxins unter Verwendung eines monoklonalen Antikörpers zeigt keinen deutlichen Vorteil gegenüber der Verwendung konventioneller polyklonaler Antikörper.

Introduction

Monoclonal antibodies, produced by classical hybridoma techniques (1, 2) offer several advantages for the purposes of radioimmunoassay (RIA), namely monospecificity and elimination of the bleed-to-bleed variation which is associated with animal antisera (3, 4). Commercial assay kits, using monoclonal antibody technology, have achieved relatively wide use in the estimation of hormones such as thyrotropin and chorionic gonadotropin because assays employing classical polyclonal antisera to these hormones display predictable cross-reaction with structurally-related molecules (5). However the role of monoclonal antibody technology in the assay of a hormone such as total thyroxine (T_4) is uncertain as there are already a large number of excellent commercial kits, utilizing polyclonal antisera, available for this purpose (6, 7).

The aim of this present study was to examine the value of using a monoclonal antibody system for the estimation of T_4 by comparing the laboratory and clinical performance of the new monoclonal antibody sectionally processed coated tube (SPAC) total T_4 kit from Mallinckrodt with that of a well established conventional T_4 assay (Amerlex: Amersham International) (8, 9).

Materials and Methods

All sera were assayed for total T_4, triiodothyronine (T_3) uptake ratio, free T_4 (FT_4) and T_3, and the free thyroxine index (FTI); computed by multiplying the T_4 concentration by the T_3 uptake ratio. Reference intervals, for all of these parameters, were determined in our laboratory.

T_4 was determined by the Amerlex (Amersham International Ltd., Amersham, Bucks, U.K.) and the MCA (SPAC) T_4 (Immunoassay Systems, Mallinckrodt, Inc. St. Louis, MO 63134) RIA kits. FT_4 and T_3 were measured with Amerlex RIA kits (Amersham International) and T_3 uptake was estimated with the MAA kit (Amersham International).

Within-batch precision of the T_4 kits was evaluated by assaying three different concentrations of a lyophilized control material 20 times in a single batch (Ortho tri-level ligand assay control, lot no. LIGX01; Ortho Diagnostics Systems, Inc., Raritan, NJ 08869). Between-batch precision was assessed by testing three concentrations of ligand once per day for seven days.

Sensitivity is defined in this study as the 'minimal detection limit' of an assay (10). It was characterised, for each T_4 assay, by using 20 replicates of a zero calibrator to calculate the concentration which corresponds to the value for counts per minute that is two standard deviations from the mean.

An association study was performed, with low, normal and high T_4 concentration sera, to determine the time needed for the reactants to reach equilibrium.

The specificity of the antibody in each T_4 kit was studied by comparing the molar ratio (expressed as a percentage) between the quantity of T_4 and the quantity of the structurally related thyroid hormones (T_3 and reverse T_3 (rT_3)) which cause 50% displacement of the T_4 tracer. T_4 and T_3 were obtained from the Sigma Chemical Company and rT_3 from Henning Berlin.

Recovery was studied by assaying aliquots of the same hypothyroid sample to which were added known and increasing amounts of T_4 standard. Recoveries were then calculated, as percentages, from the quotient of observed and calculated T_4 concentration. Parallelism and linearity were determined by assaying dilutions of a high concentration of T_4 in serum with a buffer solution containing, per litre, 1 g of bovine serum albumin, 150 mmol of NaCl, and 67 mmol of phosphate buffer, pH 7.35.

Patients

Thyroid status was determined using assays of serum FT_4, followed by assays of serum T_3 or thyrotropin (TSH) where appropriate. Subjects were then classified according to biochemical and clinical findings as follows:

Euthyroid controls: Fifty three healthy hospital and laboratory staff (20 men, 33 women).

Patients with thyroid disease: Fifteen hyperthyroid patients (4 men, 11 women) were all clinically hyperthyroid and had FT_4 concentrations > 20 pmol/l and T_3 concentrations > 2.5 nmol/l. Fifteen hypothyroid patients (2 men, 13 women) were all clinically hypothyroid and had FT_4 concentrations <10 pmol/l and thyrotropin levels >6 mU/l, T_3 values were within normal limits in nine hypothyroid patients.

Euthyroid subjects with increases in thyroxine-binding globulin (TBG) binding capacity: Third trimester pregnancy: 20 women (gestational age range 30—39 weeks). Contraceptive pill users: 20 women.

Patients on thyroxine-replacement therapy: 20 patients (2 men, 18 women). All patients were clinically controlled on thyroxine and had normal FT_4 concentrations.

Statistical analysis

The F-test was used for comparison of precision between the two methods analysed, *Student*'s paired t-test for comparison of values between both T_4 kits, and *Pearson*'s correlation coefficient (r) to show the degree of linear association among the different variables. Further statistical analyses included *Student*'s t-statistics for tests on regression coefficients (11) and the unpaired t-test for comparing means of different determinations with normal control mean values, for the various groups under study. A p value of ≤ 0.05 was considered significant.

Results and Discussion

Laboratory evaluation

Precision

The within-batch and between-batch precision for Amerlex and MCA (SPAC) T_4 kits is shown in table 1. The performance of the Amerlex kit at three different concentrations was judged to be acceptable according to the criteria of *Tonks* (12), i.e. twice the coefficient of variation (CV) should be <20%, whereas the between-batch precision for the MCA (SPAC) T_4 kit was >10% at low and normal concentrations of T_4. These variations were higher than those reported in several studies (8, 13) but similar to others (6, 7). However over the range of concentrations studies, there was no statistical difference in precision between the two methods (F-test). The ob-

served difference in precision between methods most likely arises from the inherent problems of coated-tube technology (10, 14) rather than from the nature of the reagents such as antibody type.

Tab. 1. Within-batch and between-batch precision of T_4 kits as determined with low-, medium-, and high-concentration control sera.

	Within-batch values			Between-batch values		
	Low	Medium	High	Low	Medium	High
Amerlex						
Mean (nmol/l)	30.5	89.5	193.9	27.8	93.8	201.1
SD (nmol/l)	1.4	2.4	4.2	1.4	4.6	6.2
CV (%)	4.7	2.7	2.2	5.0	4.9	3.1
MCA (SPAC)						
Mean (nmol/l)	29.9	85.0	191.0	21.5	87.1	185.2
SD (nmol/l)	3.6	6.6	10.5	2.7	9.1	6.1
CV (%)	11.9	7.7	5.5	12.8	10.4	3.3

Scatchard analysis (15), performed on data contained in the standard curve, revealed a curvilinear plot with the Amerlex antibody, indicating multiple antigenic binding sites. The calculated average affinity constant (K_a) was 6.7×10^8 l/mol. By comparison the monoclonal antibody produced a linear *Scatchard* plot, indicating a single binding site. The calculated K_a of 5.4×10^8 l/mol was comparable with a previous report for this antibody (4). The K_a values for both antibodies were similar to those reported in other RIA T_4 kits (6, 7). Although the calculated K_a value for the Amerlex antibody was slightly higher than that of the monoclonal antibody, both were of sufficiently high affinity to provide assays with *sensitivities* greater than necessary for determination of serum T_4 (minimal detectable dose for Amerlex T_4 and MCA (SPAC) T_4 were 1.5 nmol/l and 4.5 nmol/l respectively).

Association studies showed that the MCA (SPAC) T_4 method reached virtual equilibrium in the specified incubation time (one hour) at the three different T_4 concentrations studied, whereas Amerlex achieved equilibrium before 45 minutes (stated incubation time), only at a high T_4 concentration. Amerlex reactions proceeded for two hours without reaching equilibrium at normal and low T_4 concentrations.

Intermethod comparison

Using 83 serum samples, there was a high correlation ($r = 0.95$ $p < 0.001$) between T_4 levels measured in the monoclonal antibody assay and those measured in the conventional antiserum RIA. This suggests that the monoclonal antibody is directed towards the same antigenic sites on T_4 as the polyclonal antiserum. The relationship found was MCA (SPAC) $T_4 = 0.875x$ (Amerlex T_4) + 17.218. The slope of the regression line was significantly different from unity ($p < 0.001$) and the intercept statistically different from zero ($p < 0.001$), indicating that the MCA (SPAC) T_4 assay produces higher values than those of Amerlex T_4 throughout most of the assay range. In contrast, T_4 levels in low-, medium- and high-concentration control sera were lower when measured by the MCA (SPAC) T_4 assay than by Amerlex T_4 (tab. 1).

There was very little *cross-reaction* of T_3 and rT_3 in the Amerlex T_4 assay (2.7% and 1.1% respectively) and T_3 with T_4 in the MCA (SPAC) assay (0.6%). However cross-reaction of rT_3 in the monoclonal T_4 assay was high (25.0%). Since, in normal subjects, rT_3 circulated at a concentration <1% than that of T_4 (16), this cross-reaction would be unimportant in practice.

Recoveries

The Amerlex T_4 method gave a mean recovery of 98% (range 95–102%) while the MCA (SPAC) method averaged a recovery of 100% (range 91–110%). As no single recovery was less than 85% or greater than 115%, the recoveries found for both methods were acceptable according to the criterion of *Logan* (17).

The regression lines calculated for the correlation of added amount vs expected amount indicated that there were excellent linear recoveries in the monoclonal antibody and conventional antiserum RIA's ($r = 1.00$ for both assays). In addition their slopes were not significantly different from unity nor intercepts statistically different from zero (slopes and intercepts for Amerlex and MCA (SPAC) T_4, respectively, were 1.06–2.43 and 1.05, −2.25). These results indicate that there is quantitative recovery in both assays independent of the concentration of T_4 to be measured.

Test of parallelism

Amerlex and MCA (SPAC) T_4 methods produced good linearity on dilution throughout the assay range ($r = 1.00$ for both assays). The slope of the line for experimental values vs expected values was not significantly different from unity for the Amerlex assay (slope = 1.00). In contrast the MCA (SPAC) T_4 assay failed to show parallelism (slope = 0.81, $p <$

0.01). The non-parallelism, indicating interference in the monoclonal antibody assay by factors other than those which can be clearly identified by their physiochemical similarity to T_4 (10), could explain the higher MCA (SPAC) T_4 values in patient's serum compared with those determined by the Amerlex method.

Clinical evaluation

Table 2 shows means for FT_4 and results for the MCA (SPAC) and Amerlex T_4 kits in six clinical groups. The percentage of patients in these groups whose values lie outside reference intervals for FT_4 and the two T_4 kits is also shown.

Tab. 2. Serum T_4 and FT_4 values in six clinical groups.

Group	FT_4 (pmol/l)	T_4 (nmol/l)	
		Amerlex	MCA (SPAC)
Euthyroid (control) n = 53			
Mean	14.6 (2)	108.3 (4)	115.2 (4)
SD	2.2	19.6	24.7
Hyperthyroid n = 15			
Mean	47.2[c] (100)	193.2[c] (93)	181.9[c] (73)
SD	30.3	39.6	29.5
Hypothyroid n = 15			
Mean	6.5[c] (100)	47.7[c] (93)	51.9[c] (73)
SD	2.3	17.3	18.6
3rd trimester pregnancy n = 20			
Mean	9.1[c] (70)	153.0[c] (55)	162.9[c] (35)
SD	1.5	25.3	23.5
On oral contraceptives n = 20			
Mean	13.0[b] (0)	139.3[c] (30)	144.2[c] (25)
SD	2.0	19.5	21.6
On thyroxine replacement therapy n = 20			
Mean	16.1 (0)	110.7 (0)	115.2 (0)
SD	3.4	18.3	23.6

Statistical significance of difference from euthyroid (control) mean indicated by [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$. Numbers in parentheses are the percentages of patients whose values lie outside the reference interval by each measure.

Patients with thyroid disease

The mean values for T_4 by both methods – as expected – were increased ($p < 0.001$) in hyperthyroidism. In this group one patient (7%) had a normal T_4 when measured by Amerlex whereas T_4 values assayed by the MCA (SPAC) kit were within the euthyroid range in four patients (27%). Amerlex and MCA (SPAC) T_4 values were significantly decreased ($p < 0.001$) in hypothyroid patients. These patients were distinguished with 93% accuracy from normal subjects by the Amerlex T_4 kit (tab. 2) whereas only 73% of hypothyroid patients had MCA (SPAC) T_4 values below normal limits.

Thus T_4 values measured by the conventional antibody RIA were a slightly more sensitive indicator of thyroid disease than those assayed by the monoclonal antibody assay.

Subjects with increased thyroid hormone binding capacity

The concentration of TBG progressively increases with the duration of *pregnancy* (18, 19). Therefore – as expected – T_4 values by both methods were significantly increased ($p < 0.001$) as compared with the control group in third trimester pregnants. In these women, more than one-half (55%) of their T_4 values by Amerlex were in the hyperthyroid range whilst about a third (35%) of T_4 values assayed by MCA (SPAC) were elevated.

In *oral contraceptive users*, T_4 values by both methods were increased ($p < 0.001$), as a direct consequence of elevated TBG concentrations. Amerlex and MCA (SPAC) T_4 values were elevated in 30% and 25% of patients on oral contraceptives, respectively.

Therefore, in sera of pregnant women and oral contraceptive users, the T_4 value was a more appropriate reflection of TBG concentration, when measured by the conventional RIA than by the monoclonal antibody assay. All 20 *patients controlled on thyroxine-replacement therapy* gave normal T_4 values by Amerlex and MCA (SPAC) methods. Hence concentrations measured by the monoclonal antibody assay and by the conventional antibody RIA appeared to be of equivalent value in monitoring thyroxine-treated patients.

Correlation between FTI and FT_4

There were good correlations between FT_4 concentration and FTI values derived from each of the T_4 methods in euthyroid control subjects and patients

with thyroid disease (Amerlex, $r = 0.81$; $p < 0.001$ and MCA (SPAC), $r = 0.78$; $p < 0.001$). Thus FTI values derived from either the conventional or the monoclonal antibody T_4 assay, were equally good in reflecting a patient's true thyroid status.

Conclusions

In conclusion, the monoclonal antibody used in the Mallinckrodt (SPAC) T_4 had a similar affinity constant to that of a polyclonal antiserum and showed a clinically insignificant but high cross-reaction with the structurally-related thyroid hormone, rT_3. In addition, the assay showed good sensitivity, quantitative recoveries and technical simplicity. Furthermore it demonstrated acceptable diagnostic accuracy, despite the slightly lower sensitivity of the assay as an index of thyroid status in thyroid disease, compared with that of a polyclonal antibody RIA. Therefore, in conclusion, a monoclonal antibody T_4 assay is suitable for use in the clinical laboratory, but it offers no significant advantages over a RIA employing a conventional polyclonal antiserum.

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